Journal of Physiology (1989), **413**, *pp*. 521–541
With 11 *text-figures*
Printed in Great Britain

THE EFFECT OF THE PHENYLALKYLAMINE D888 (DEVAPAMIL) ON FORCE AND Ca^{2+} CURRENT IN ISOLATED FROG SKELETAL MUSCLE FIBRES

BY RITA ERDMANN AND H. CH. LÜTTGAU
From the Department of Cell Physiology, Ruhr-University, D-4630 Bochum, FRG

(*Received 4 October 1988*)

SUMMARY

1. The effects of the (+)- and the (−)-isomer of the phenylalkylamine derivative D888 (desmethoxyverapamil or devapamil) on isometric force and slow Ca^{2+} inward current were investigated in short toe muscle fibres of the frog (*Rana temporaria*). The experiments were performed under voltage-clamp conditions with two flexible internal glass microelectrodes at 10 °C in a TEA sulphate solution containing ~ 4 mM-free Ca^{2+}.

2. In the presence of 0·05–5 μM-(−)-D888 a normal phasic contracture could be induced by a depolarizing voltage step. When depolarization was maintained for some minutes the force-controlling system turned into a stabilized inactivated state (paralysis) from which it recovered upon repolarization within minutes instead of seconds. With the (+)-isomer (0·5–20 μM), a similarly retarded restoration was observed. However, it proved to be less effective than the (−)-isomer.

3. D888 caused a shift to more negative potentials of the S-shaped curve, which describes the voltage dependence of force restoration in the steady state (restoration time 15 min). The potential of half-maximum restoration in the absence of the drug ($\bar{V} = -35{\cdot}8$ mV) changed as follows. (−)-D888: −56 mV (0·05 μM), −69 mV (0·2 μM), −77·5 mV (0·5 μM), and −82 mV (5 μM); (+)-D888: −55·8 mV (0·5 μM), −76·5 mV (5 μM), and −85 mV (20 μM).

4. On the assumption that D888 binds only to the inactivated form of the voltage sensor of force control in the T-tubular membrane (modulated receptor hypothesis) the data presented in paragraph 3 allowed an estimation of the drug-receptor dissociation constants. The K_D values ascertained in this way, 1·71 nM for the (−)-isomer and 12·9 nM for the (+)-isomer, are in fair agreement with those obtained from [^{3}H]D888 binding studies by other authors.

5. A comparison between equal concentrations of the two isomers regarding their effect on the speed of restoration and the time needed to transform the sensor into the paralysed state suggests that the differences in the dissociation constants are mainly due to a greater dissociation rate of the (+)-isomer from the sensor.

6. The restoration of the Ca^{2+} channel was retarded by D888 to a similar extent as that of the voltage sensor. This parallel action on both systems indicates structural similarities between the voltage sensor and the Ca^{2+} channel.

7. It is concluded that D888 'stabilizes' the inactivated state of the voltage

sensor and the Ca^{2+} channel in a way similar to D600, but with a higher potency. Both isomers of D888 showed an antagonistic action and differed only in their potency.

INTRODUCTION

In the presence of gallopamil (D600), a member of the phenylalkylamine class of Ca^{2+} antagonists, skeletal muscle fibres change to a mechanical refractory state after one normal phasic contracture (Eisenberg, McCarthy & Milton, 1983). They slowly recover from this state, which has been defined as paralysis, during a long-lasting hyperpolarization. The stabilization of the force controlling voltage sensor in the T-tubular membrane in the refractory state has been explained by Berwe, Gottschalk & Lüttgau (1987) with a state-dependent binding of the drug to the inactivated sensor (modulated receptor hypothesis, cf. Hille, 1977). Measurements of intra-membrane charge movements, supposed to reflect the gating of the voltage sensor, have directly confirmed the concept of a state-dependent interaction of the drug with this system (Hui, Milton & Eisenberg, 1984; Melzer & Pohl, 1987).

Here we quantitatively describe the effect of D888 (devapamil), a phenyl-alkylamine derivative with a higher potency than D600 (Nawrath & Raschack, 1987), on force and slow Ca^{2+} inward current in short toe muscle fibres. The central part of this study is the analysis of the effect of each of the two isomers of this drug on the steady-state potential dependence of force restoration. From the shift in the mid-point voltage of the restoration curve at different concentrations we directly derived the dissociation constants for both isomers by assuming a Boltzmann distribution of the charged sensor in the T-tubular membrane and a selective binding to the inactivated state. The K_D values ascertained in this way agreed satisfactorily with those obtained from [^{3}H]D888 binding studies, i.e. with an entirely different method (e.g. Goll, Ferry, Striessnig, Schober & Glossmann, 1984). The time course of the slow Ca^{2+} inward current was altered by the drug in a way comparable to that of the sensor, suggesting structural similarities between the two systems.

Preliminary accounts of some of the experiments described here were presented at the Cambridge meeting of the Physiological Society (Erdmann, Lüttgau & Melzer, 1988).

METHODS

Preparation

Healthy specimens of the frog *Rana temporaria* were killed by decapitation and the m. lumbricalis digiti IV of each hindlimb was dissected. The experiments were performed with small bundles of muscle fibres from this muscle. Isometric force of a single fibre (average length, 1·3 mm) was measured under voltage-clamp control with two flexible internal microelectrodes as described in detail elsewhere (Gomolla, Gottschalk & Lüttgau, 1983). A uniform depolarization under voltage-clamp conditions was achieved by replacing Ringer solution with a solution containing TEA (tetraethylammonium) sulphate as the main solute (Berwe & Feldmeyer, 1984).

Solutions

Standard Ringer solution A. This solution contained (in mM): NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85.

Sulphate Ringer solution B. This solution contained (in mM): Na_2SO_4, 38·75; K_2SO_4, 1·25; $CaSO_4$, $\sim$ 9·4; Na_2HPO_4, 1·08; NaH_2PO_4, 0·43; sucrose, 113.

Tetraethylammonium sulphate solution C. This solution contained (in mM): $(TEA)_2SO_4$, 40; K_2SO_4, 1·25; $CaSO_4$, ~ 9·4 (free Ca^{2+} ~ 4 mM); MOPS (3-[*N*-morpholino]propanesulphonic acid), 5; sucrose, 113.

The solutions B and C also contained 10^{-7} g/ml TTX (tetrodotoxin). The pH of all solutions was 7·0. The isomers of the Ca^{2+} antagonist D888 (devapamil) were from Knoll AG, Ludwigshafen, FRG. They were usually added to solution C from a 1 mM stock solution.

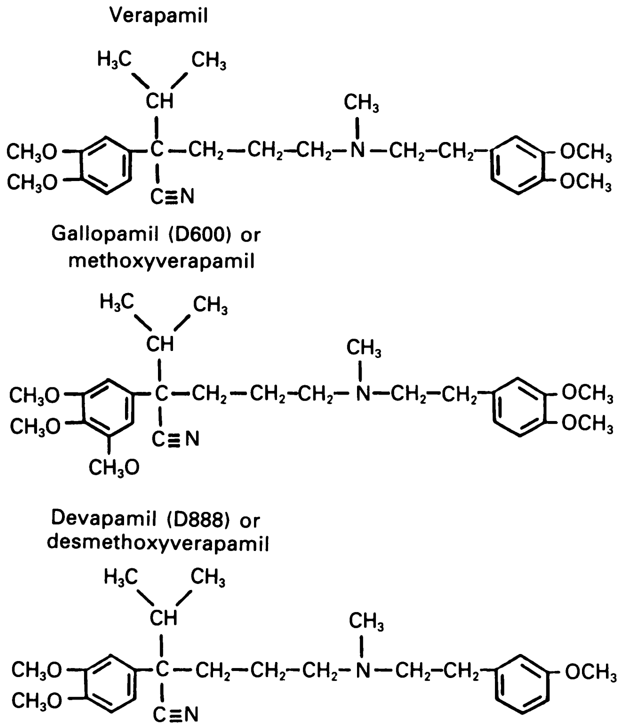

Fig. 1. Verapamil and its derivatives gallopamil (methoxyverapamil, D600) and devapamil (desmethoxyverapamil, D888).

General procedure

After the muscle preparation had been transferred to the experimental chamber on a piece of Sylgard, solution A was first replaced by solution B (sulphate Ringer solution). This caused a transient depolarization with force development induced by the removal of external Cl^- and a subsequent efflux of internal Cl^-. After relaxation, solution B was replaced by the TEA sulphate solution (solution C) which occasionally caused a second transient force development. Ultimately, the drug was applied after complete relaxation had occurred. First impalements were attempted 30 min later. Thus, the described experiments with D888 were performed between about 45 and 200 min after the application of the drug. Most contracture experiments were done with 2 mM-Cd^{2+} in solution C (added from a 2 M-$CdCl_2$ solution).

Temperature

The muscles were dissected in a pre-cooled standard Ringer solution and the experiments were mostly performed in solution C at $10 \pm 0·2$ °C if not stated otherwise.

Fitting procedure

For least-squares fitting we made use of the so-called MINPACK library (More, Garbow & Hillstrom, 1980). The FORTRAN source was provided by the Ruhr-University Computation Center and modified for use on our laboratory minicomputer (SMS 1000, Scientific Micro Systems Inc. with processor LSI 11/73 and RT 11 operating system, Digital Equipment Corporation).

RESULTS

Initial experiments

In order to get a first survey of the mode of action and potency of D888 and also of possible differences in these qualities between the two isomers, we investigated the process of restoration after a long-lasting depolarization. This approach was chosen because a retardation in force restoration was always observed as a characteristic and sensitive signal after applying D600, the methoxy derivative of verapamil (Berwe *et al.* 1987).

Following the general procedure at the beginning of each experiment, which has been described in detail under Methods (exchange of solutions, observance of waiting periods), a fibre was selected, impaled and clamped at −90 mV for 5 min, the usual conditioning time (Fig. 2*B*). In this experiment, the TEA sulphate solution (solution C) without Cd^{2+} contained 2 μM-(+)-D888. The fibre was subsequently depolarized to 0 mV and developed a normal phasic contracture. The spontaneous relaxation after about 5 s reflects the transition of the force-controlling system (voltage sensor) in the T-tubular membrane into a refractory or inactivated state. After 3·5 min, the fibre was repolarized to −90 mV to restore the contraction ability. However, a second depolarization after 1·5 min revealed that no detectable restoration had taken place, indicating that the fibre was in the paralysed state. In the absence of the drug, a full restoration could be observed with exactly the same pulse programme (Fig. 2*A*). In former control experiments, Berwe *et al.* (1987) found that, under roughly the same conditions, maximum force could even be obtained within only 20 s. In the control experiment we repeated the pulse sequence 4 times and observed slightly decreasing contractures and a long-lasting afterphase following the 4th contracture. In the presence of the drug (Fig. 2*B*), 5 min at −120 mV eventually restored the ability to develop force as revealed by the third depolarization. Following the concept developed to explain the action of D600 (Berwe *et al.* 1987), we assume that D888 can only bind to the inactivated voltage sensor (I), thus transferring it into a stable secondary inactivated state (I_0).

$$\mathrm{R} \longleftrightarrow \mathrm{A} \longleftrightarrow \mathrm{I} \xleftrightarrow[\text{D888}]{} \mathrm{I_0}$$

(R and A are the sensor in the resting and active state, respectively.) Figure 2*C* shows that in the presence of a relatively low concentration (0·5 μM) of the (−)-isomer paralysis could be induced, revealing the same mode of action as observed with the (+)-isomer. However, a restoration time of 5 min at −120 mV was not sufficient for maximum force to be reached so that a second restoration period became necessary for complete restoration.

It is known from the early experiments with D600 (Eisenberg *et al.* 1983) that the recovery from paralysis is highly temperature dependent. At a temperature of 22 °C, 30 μM-D600 proved to be inefficient to induce paralysis. Having these results in mind, we performed a few high-temperature experiments with the pulse programme just described. We applied 0·2 and 0·5 μM-(−)-D888 at a temperature of 20 °C. In both cases paralysis was complete. However, the speed of restoration was accelerated considerably. In the presence of 0·5 μM-(−)-D888 restoration was complete after 5 min at −120 mV while this time was insufficient at 10 °C (see Fig. 2*C*). In agreement with Siebler & Schmidt (1987), who investigated the temperature dependence of the effect of D600, we can state that temperature has a great effect on the speed of restoration in the presence of phenylalkylamine derivatives. However, the effects

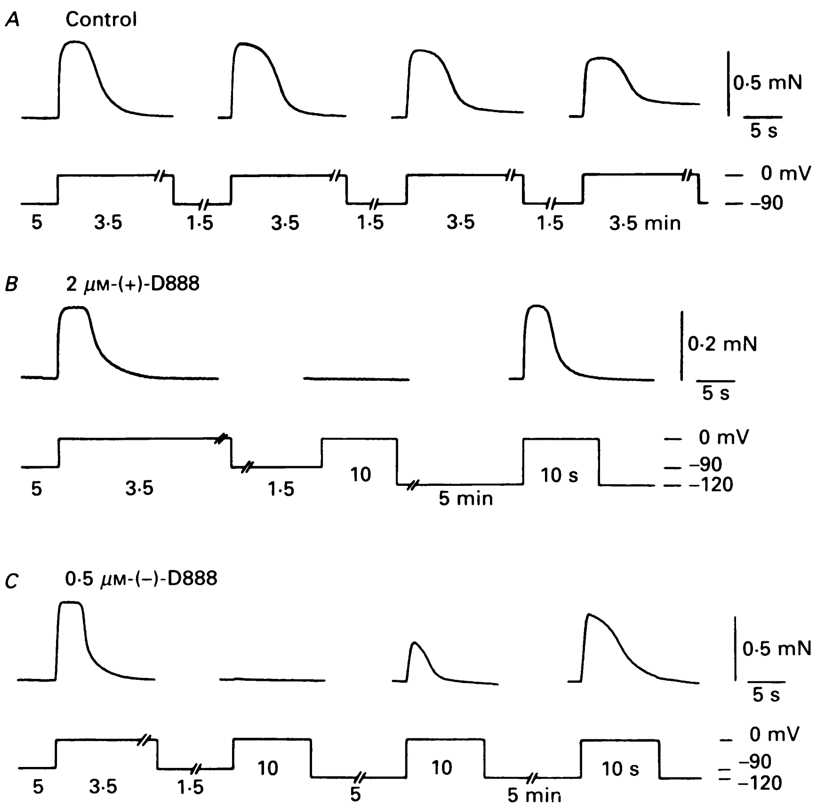

Fig. 2. Restoration after long-lasting depolarizations in the absence (*A*, control) and in the presence of 2 μM-(+)-D888 (*B*) and 0·5 μM-(−)-D888 (*C*). The pulse programme is indicated below each record. Three different fibres; solution C without Cd^{2+}; temperature = 10 °C.

of (−)-D888 at low and high temperature differ in a quantitative rather than a qualitative manner.

Time course of restoration

The extent and speed of restoration depend on membrane potential. Applying voltage-clamp steps Berwe *et al.* (1987) found that at holding potentials of −120 and −90 mV maximum force was regained within 5–7 s and about 30 s, respectively (6·7 °C). Since the experiments described in the last section revealed that D888 retards the restoration process, we tried to measure the time course of restoration in a more quantitative way with short test pulses.

The experiments were usually performed in the following way (see Fig. 3): after two test pulses from a holding potential of −120 or −100 mV, the fibre was depolarized for 3 min. As will be shown later, this duration is sufficient to turn the system into a paralysed state (I_0) as long as the concentrations of the isomers remain distinctly higher than their respective dissociation constant. (The dissociation

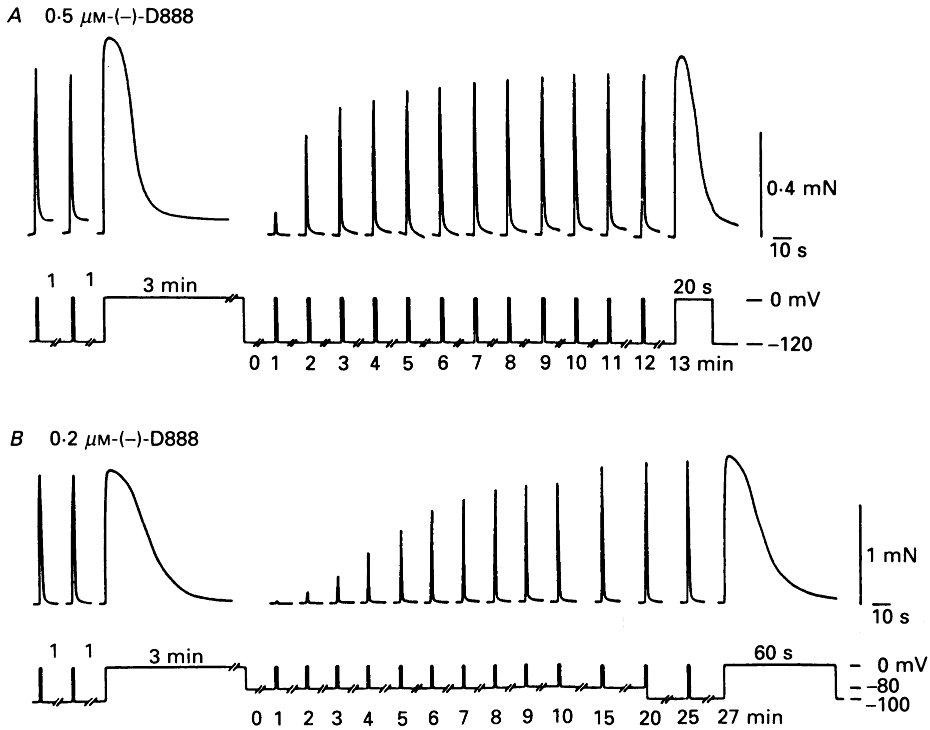

Fig. 3. Two examples of the restoration rate following the transformation into the paralysed state by a depolarizing pulse of 3 min duration. The pulse programme, which included short depolarizing pulses of 1 s duration, can be seen below each record. Solution C with 2 mM-Cd^{2+} and 0·5 μM- (*A*; HP = −120 mV) or 0·2 μM-(−)-D888 (*B*; HP = −80 mV), respectively. Two fibres; temperature = 10 °C.

constants obtained from binding studies are given in Table 1). Afterwards the fibre was repolarized to the selected holding potential and force restoration was tested every minute by a short depolarizing pulse of 1 s. Maximum force induced by the test pulse was usually slightly less than that induced by longer-lasting depolarizations (see Fig. 3), indicating that a 'saturation' had not yet been reached. The pulses retarded the restoration process. This became clearly discernible when the holding potential was close to the potential where the steep part of the steady-state restoration curve begins (cf. Fig. 3*B*). Therefore, we often interrupted the pulse sequence after 10 min and induced the subsequent pulses every 5 min. Finally, we checked the quality of the fibre by inducing force with a longer-lasting depolarizing pulse.

In these and further experiments we added 2 mM-Cd^{2+} to the solution without reducing the Ca^{2+} content. Cadmium at least partially prevented the increase in leakage current and prolonged the lifetime of the fibres under voltage clamp. In

Fig. 3, restoration time curves at a holding potential close to the resting potential (−80 mV) and at a more negative potential (−120 mV) can be seen. At −120 mV a relatively fast restoration was observed during the first 3 min, followed by a slower restoration to the maximum (Fig. 3*A*). At −80 mV restoration proceeded slowly

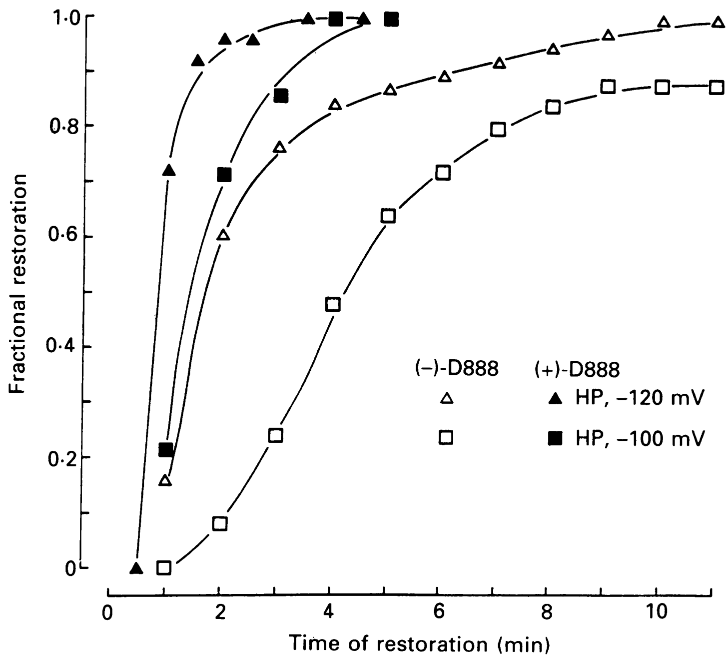

Fig. 4. Comparison of the effect of either of the two isomers on the time curves of restoration at −120 and −100 mV. Restoration was preceded by a depolarizing pulse of 3 min duration to induce paralysis. Each curve corresponds to an experiment with one fibre. Three fibres were stimulated once a minute and the fourth at one pulse every 30 s. Solution C with 2 mM-Cd^{2+} and 0·5 μM of the (+)- or the (−)-isomer of D888, respectively; temperature = 10 °C.

from the beginning and maximum force was eventually reached after the pulse frequency had been reduced (Fig. 3*B*). In this case, the holding potential was identical with the most positive potential at which, in the steady state, maximum force could be restored. In further experiments, we confirmed that even under unfavourable conditions, as in Fig. 3*B*, a steady state in force could be reached at least within 15 min.

Figure 4 compares the speed of restoration at −120 and −100 mV in the presence of 0·5 μM of the (−)- and the (+)-isomers of D888. At both potentials restoration was faster if the (+)-isomer was present. With a holding potential of −120 mV half-maximum restoration was reached within 51 and 109 s with the (+)- and the (−)-isomer, respectively. The corresponding values for −100 mV were 87 s for the (+)- and 240 s for the (−)-isomer.

The process of restoration could be accelerated by the application of caffeine. In the experiment presented in Fig. 5, we compared the speed of restoration of a paralysed fibre in the presence of 0·5 mM-caffeine with that of another in the absence of this drug. The solution contained, in both cases, 0·5 μM-(−)-D888. Caffeine did not induce a spontaneous contracture. However, it influenced the speed of restoration. In the presence of caffeine about 2 min were sufficient to reach 80% of maximum force while in its absence about 6–7 min were needed. Caffeine is known to activate the Ca^{2+}-release channel of the sarcoplasmic reticulum (Rousseau, LaDine, Liu &

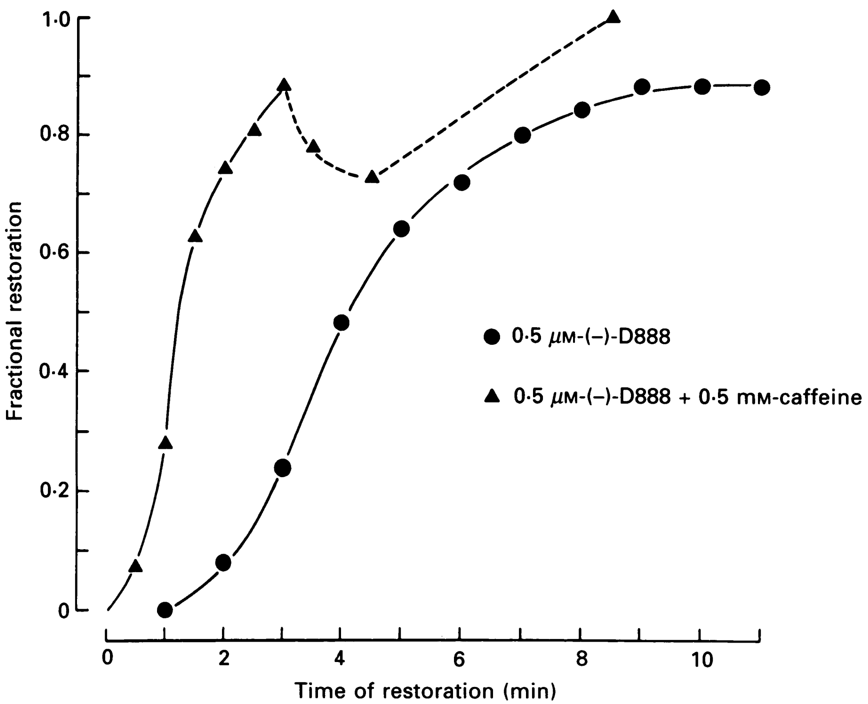

Fig. 5. The effect of 0·5 mM-caffeine on the speed of restoration at 0·5 μM-(−)-D888. The fibres were paralysed during an initial depolarizing pulse of 3 min; holding potential during restoration, −100 mV. Notice different stimulation rates. Stimulus duration, 1 s. In the presence of caffeine, the decline in force after 3 min (start of the dashed line) is probably related to the high stimulation rate of 1 every 30 s since maximum force was ultimately reached after a rest of ~ 4 min. Two fibres; solution C with 2 mM-Cd^{2+}; temperature = 10 °C.

Meissner, 1988). Thus, a slight caffeine-induced increase in internal free Ca^{2+}, insufficient to generate detectable force, might have caused the observed acceleration in force restoration by a direct effect of Ca^{2+} on the gating mechanism of the voltage sensor (cf. Lüttgau, 1970). Besides this interpretation favoured by us the results could also be explained by assuming that caffeine acts directly on both the Ca^{2+}-release channel and the voltage sensor. The curve obtained with caffeine reveals a decline after 90% of maximum force had been reached. This was certainly related to the high stimulation rate (1 every 30 s), since after an interruption of stimulation for 4 min force reached its maximum. An explanation for this secondary effect of caffeine cannot be given.

Speed of sensor transformation from the inactivated into the paralysed state

The experiments described so far show that the transition from the inactivated into the paralysed state takes place rather slowly. However, experiments like those described in Fig. 2 do not provide detailed information. Therefore, the following pulse programme (Fig. 6) was designed to measure more directly the speed at which the sensor shifts from I to I_0. The fibre was depolarized from -90 mV to zero and

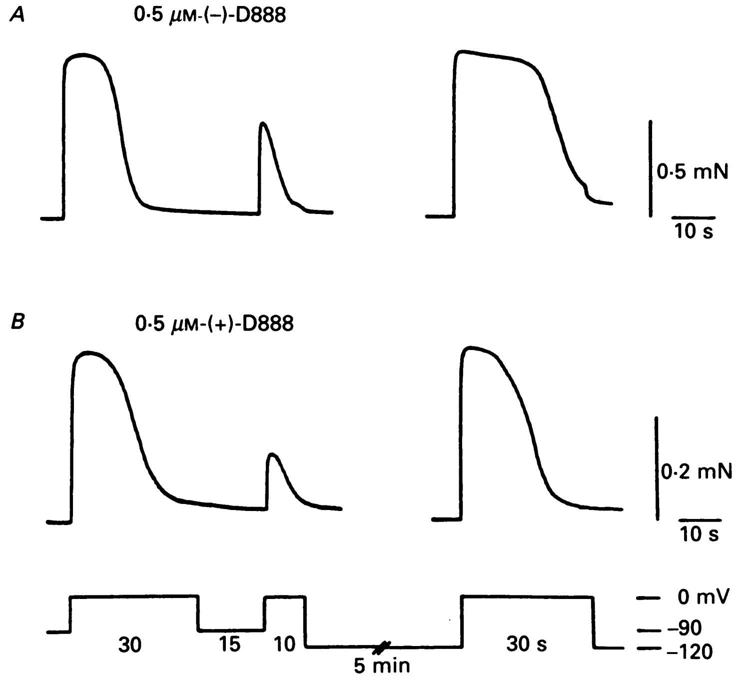

Fig. 6. Example of the experimental procedure for measuring the speed of sensor transformation from the inactivated into the paralysed state. The pulse programme for both records is shown below. For further explanations see text. Two fibres; solution C with 2 mM-Cd^{2+} and 0·5 μM of the (−)- (*A*) and the (+)-isomer (*B*), respectively; temperature = 10 °C.

after varying periods of time, when spontaneous relaxation had occurred, it was repolarized back to -90 mV for 15 s. In the absence of the drug *ca* 15 s at a moderate negative holding potential are needed to regain the ability to develop maximum force (Berwe *et al.* 1987; cf. Fig. 11), i.e. to shift inactivated sensors in the resting state, while this time is insufficient to transform a significant amount of paralysed sensors into the resting state. As a consequence, the force developed at the end of the repolarization pulse approximately reflects that percentage of the system which was not yet in the paralysed state when the test pulse started. Figure 6*A* thus shows that after a depolarization time of 30 s a considerable amount of the system was still in the inactivated state, even when 0·5 μM of the (−)-isomer had been applied. This concentration is roughly 300 times larger than the dissociation constant of the (−)-isomer (see Table 1). Surprisingly, the situation was very similar when the same concentration of the (+)-isomer was tested (Fig. 6*B*), which is, however, only 40 times larger than the K_D of the (+)-isomer.

In further experiments with this pulse programme we observed a great divergence of data. In addition, the restoration time curves suggest that, at least in the presence of low concentrations of the (+)-isomer, a small amount of restoration from I_0 to R might have taken place during the 15 s pulse. Therefore, we did not attempt a quantitative evaluation of the forty tests which we performed with this pulse programme at different concentrations. Qualitatively, the results can be summed up as follows: (1) The transition into the paralysed state was a relatively slow process. With 0·5 μM of either isomer about 90–120 s were needed until a response upon the 15 s pulse could no longer be detected. (2) A depolarization of 3 min appeared to be sufficient to transform the sensor into the paralysed state, even when the isomers were present at low concentrations. Only with the lowest concentration applied, i.e. 0·05 μM of the (−)-isomer, was paralysis as estimated in the described way mostly not complete. Force induced at the end of the test pulse varied between 0 and 58% of the maximum. (3) When the same concentration was used, the (+)-isomer proved to be as effective as the (−)-isomer in transforming the sensor into the paralysed state. With 0·5 μM of either isomer force still reached about 50% after 60 s and was always absent after 90–120 s. With 5 μM of the (+)-isomer, which was as effective as 0·5 μM of the (−)-isomer in shifting the steady-state restoration curve to more negative potentials (cf. Figs 7 and 8), complete paralysis was reached after 30 s.

Steady-state dependence of force restoration on the holding potential

A state-dependent binding of D888 to the inactivated sensor reduces the probability of the system being in the resting state and should result in a shift to negative potentials of the steady-state potential dependence of force inactivation (cf. Hille, 1977). This has recently been shown to be the case with D600, another verapamil derivative (Berwe *et al.* 1987). Here we carried out related experiments with both isomers of D888.

The experiments were performed with a pulse programme roughly identical to that shown in Fig. 3. At a constant holding potential of −100 mV, we initiated two short contractures by 1 s pulses with a time interval of 60 s. Afterwards a long-lasting depolarizing pulse of 3 min duration followed to paralyse the fibre. At the end of this time the rate of restoration was investigated, again with short pulses at an interval of 60 s, from the respective holding potential. Subsequent to the 10th pulse, the stimulation interval was prolonged to 5 min in order to minimize the retarding effect on restoration of the test depolarizations, before a further contracture was induced after a total restoration time of 15 min. Afterwards the fibre was hyperpolarized to −120 mV and a control contracture was elicited after 5 more minutes. Maximum force after 15 min was regarded as the steady-state value at the corresponding holding potential and the concentration of the drug used. It was compared with that after 5 min at −120 mV as the 100% value. In a few experiments, hyperpolarization to −120 mV was no longer appropriate because of a drastic increase in the holding current. In this case, the second pulse prior to paralysis was used as the 100% control value. We always added 2 mM-Cd^{2+} to the external solution in order to stabilize the fibre membrane.

To obtain control data in the absence of the drug we applied the same pulse programme as described above. However, since the fibres were in the inactivated and not in the paralysed state after a depolarization time of 3 min, restoration was obtained much more quickly. At rather positive holding potentials, however, we

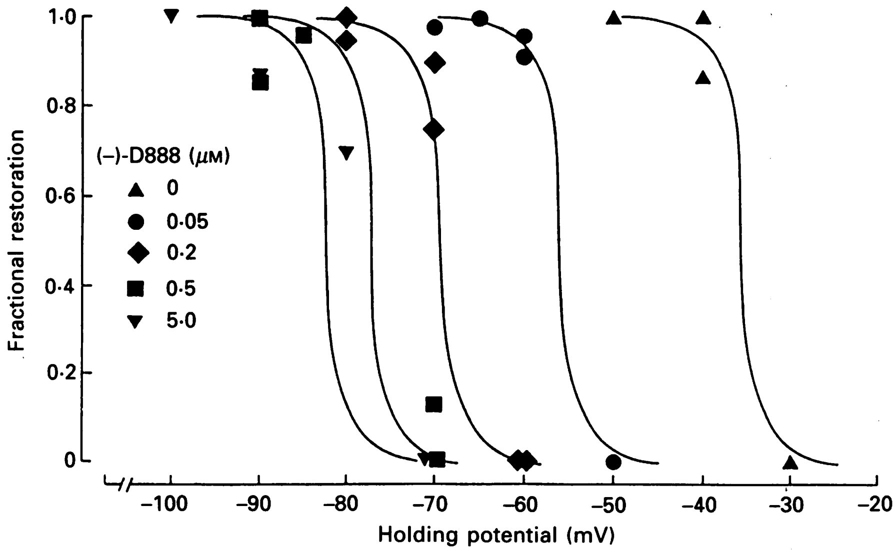

Fig. 7. Shift in the steady-state potential dependence of force restoration to more negative potentials at different concentrations of (−)-D888. The extent of force regained after 15 min of restoration is plotted on the ordinate as fraction of maximum force *vs.* the holding potential during restoration on the abscissa. The concentrations applied are shown in the inset. For further details see text. The differences between the mid-point voltage $\bar{V}$ of the control curve (−35·8 mV) and those in the presence of (−)-D888 (e.g. $\Delta\bar{V}$) are as follows: 20·2 mV (0·05 μM), 33·2 mV (0·2 μM), 41·7 mV (0·5 μM), and 46·2 mV (5 μM). Each point corresponds to an experiment with one fibre. Solution C with 2 mM-Cd^{2+}; temperature = 10 °C.

repeatedly observed a phenomenon already described by Berwe *et al.* (1987), i.e. an increase in force at the beginning of restoration followed after several minutes by a steady decline. The latter was explained by the cited authors as a slow transition into a state similar to paralysis even in the absence of the Ca^{2+} antagonists. This was not due to a deterioration of these fibres, because they recovered completely during a hyperpolarizing period. Since we were interested in a control curve from non-paralysed fibres, we always evaluated the maximum value registered in the course of the restoration period. In the presence of D888, we did not observe this phenomenon.

The steady-state curves at different drug concentrations, obtained in the described way, are shown in Fig. 7 for the (−)- and in Fig. 8 for the (+)-isomer. The extent of force restoration regained during the restoration time of 15 min is plotted on the ordinate as the fraction of maximum force *versus* the holding potential during the restoration period on the abscissa. Every point is the result of a successfully finished restoration pulse sequence with one individual fibre. Since only a few data were available for each curve, it was difficult to estimate exactly its steepness. However, the results show that at all concentrations applied the potential dependence was comparably steep. We, therefore, decided to use the same steepness for all curves, i.e. to adapt each curve to the available data by a parallel shift in the control curve.

The mid-point voltage ($\bar{V}$) of the control curve was ~ -40 mV (We used the same control data for both isomers.). This rather positive value, as compared with that measured in former experiments (e.g. Berwe *et al.* 1987), may be explained by the

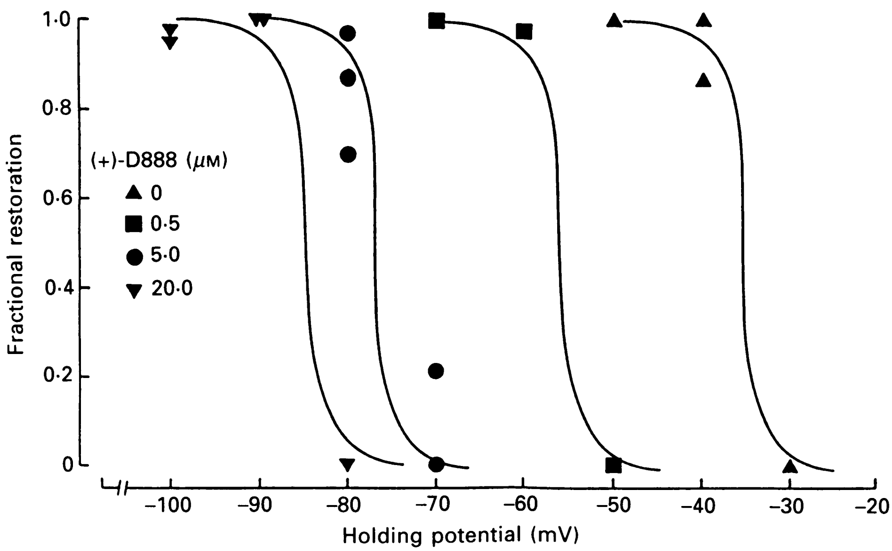

Fig. 8. Shift in the steady-state potential dependence of force restoration to more negative potentials at different concentrations of (+)-D888. Same procedure as in Fig. 7. The difference between the mid-point voltage $\bar{V}$ of the control curve ($-35{\cdot}8$ mV) and those in the presence of D888 (e.g. $\Delta\bar{V}$) are as follows: 20 mV (0·5 μM), 40·7 mV (5 μM), and 49.2 mV (20 μM). Solution *C* with 2 mM-Cd^{2+}; temperature = 10 °C.

additional screening of external surface charges with 2 mM-Cd^{2+}. The application of D888 caused a negative shift in the restoration curve. The increment in shift became smaller with higher concentrations, suggesting a logarithmic rather than a linear dependence of the mid-point voltage on concentration. As expected, the (−)-isomer proved to be more effective than the (+)-isomer. For example, a roughly equal shift was obtained with 0·05 μM of the (−)-isomer and 0·5 μM of the (+)-isomer or with 0·5 μM of the (−)-isomer and 5·0 μM of the (+)-isomer. Thus, the (−)-isomer was roughly 10 times more effective than the (+)-isomer.

The steady-state curves shown in Figs 7 and 8 are much steeper than, for example, those for the steady-state 'inactivation' of charge movements (Brum, Fitts, Pizarro & Rios, 1988) or the steady-state inactivation of Ca^{2+} release (Brum, Rios & Stefani, 1988). This, however, is not surprising since restoration of force only becomes detectable when Ca^{2+} release has passed a 'threshold' and reaches a maximum (saturation of Ca^{2+} binding sites of troponin), while maximum restoration of Ca^{2+} release will finally be obtained at more negative holding potentials. We suppose that a steady state was always approached within 15 min. However, we cannot completely exclude the possibility that further restoration might occur if it proceeds with a slower time scale.

For the interpretation and subsequent evaluation of the restoration curves shown in Figs 7 and 8 we assume a voltage-dependent Boltzmann distribution of the charged sensor between a resting and therefore activatable state and an inactivated one. In the steady state, the probability of finding the system in the resting state (P_R) is then given by the following equation:

$$P_{\mathrm{R}} = \frac{1}{1+\exp\,[(V-\bar{V})/k]}, \tag{1}$$

with V being the restoration holding potential, $\bar{V}$ the mid-point voltage of half-maximum restoration and k a steepness factor. A selective binding of D888 to the inactivated sensor (modulated receptor hypothesis, Hille, 1977), as previously suggested to explain the action of D600 (Berwe *et al.* 1987), should reduce the probability of finding the sensor in the resting state and should cause a shift in the steady-state restoration curve to more negative potentials (cf. Bean, 1984). The potential of half-maximum restoration in the presence of D888 then equals

$$\bar{V}' = \bar{V} - k\ln\left(1+\frac{S}{K_{\mathrm{D}}}\right), \tag{2}$$

with S being the concentration of the drug and K_D the dissociation constant of the D888–receptor complex. The shift of the restoration curve ($\Delta\bar{V}$) in the presence of different concentrations of (−)- and (+)-D888 can then be determined by rewriting equation (2) as follows:

$$\Delta\bar{V} = k\ln\left(1+\frac{[(-)\text{-D888}]}{K_{\mathrm{D}(-)}}\right), \tag{3}$$

$$\Delta\bar{V} = k\ln\left(1+\frac{[(+)\text{-D888}]}{K_{\mathrm{D}(+)}}\right). \tag{4}$$

Taking the $\Delta\bar{V}$ values for different concentrations of both isomers of D888 obtained from the curves shown in Figs 7 and 8 and given in the legends of these figures, a least-squares fit was carried out of eqns (3) and (4) to these data. The fit provided the following values for the free parameters in the fit: $k = -6{\cdot}47$ mV, $K_{D(-)} = 1{\cdot}71$ nM and $K_{D(+)} = 12{\cdot}9$ nM.

In Fig. 9 we plotted the measured shifts of the mid-point voltage ($\Delta\bar{V}$) *versus* ln $(1+S/K_D)$. In the latter term the ascertained K_D values for the (−)- and the (+)-isomer, respectively, were inserted. The figure shows that a single line can reasonably well describe the dependence of $\Delta\bar{V}$ on the logarithmic term, i.e. this simple model can sufficiently describe the shift of $\bar{V}$ over a large concentration range.

The restoration time curves described above (Fig. 4) revealed that restoration developed faster in the presence of the (+)-isomer. Since the speed of transforming the sensor into the paralysed state was roughly equal if the (+)- or the (−)-isomer was present it appears most likely that the roughly 10 times larger dissociation constant of the (+)-isomer, as estimated here, is mainly due to a larger dissociation rate constant of this isomer.

The Ca^{2+} current in the presence of (−)-D888

Depolarization is accompanied by a Ca^{2+} inward current through a fast and a slow Ca^{2+} channel (cf. Cota & Stefani, 1986). In this section we describe the effect of D888 on the slow (L-type) Ca^{2+} channel (Sanchez & Stefani, 1978) during a paralysis-inducing pulse programme. The current flowing through the fast Ca^{2+} channel (Cota & Stefani, 1986) could not be resolved.

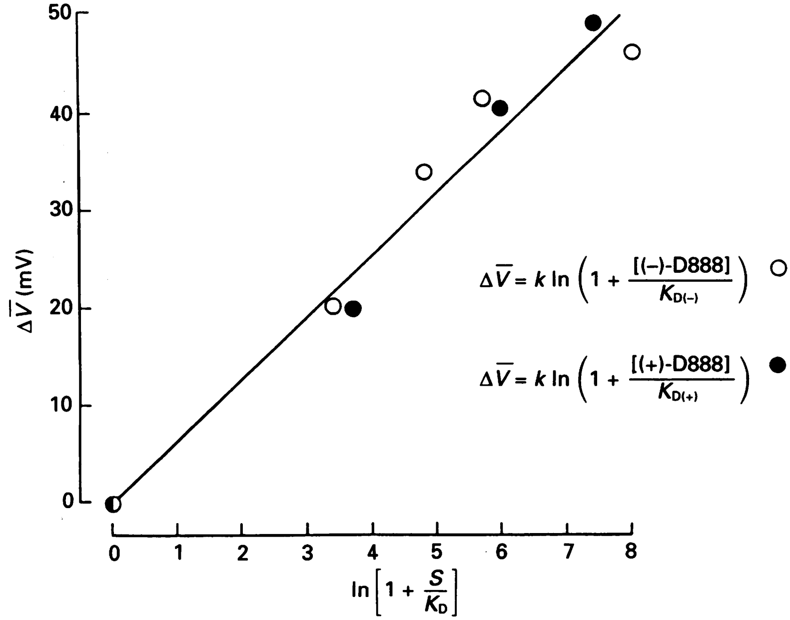

Fig. 9. Shift in mid-point voltage $\Delta\bar{V}$, derived from the restoration curves shown in Figs 7 and 8, *vs.* $\ln(1+S/K_D)$. For K_D the corresponding values gained from the fitting procedure were inserted in the term on the abscissa. ○, (−)-D888; ●, (+)-D888. The straight line is the graphic presentation of the equations shown in the inset with the values for the 'free' parameters k, $K_{D(-)}$ and $K_{D(+)}$ obtained from the fit. Correlation coefficients of the measured $\Delta\bar{V}$ values: (−)-isomer, $R^2 = 0{\cdot}96$, $k_1 = -6.17$ mV; (+)-isomer, $R^2 = 0{\cdot}99$, $k_2 = -6{\cdot}82$ mV.

In the control experiment shown in Fig. 10, we depolarized the fibre for 3 min and induced a normal phasic contracture. In the current record, a short-lasting outward current was followed by a transient inward current which we regarded as the slow Ca^{2+} current. It had the same time course as the Ca^{2+} current described by Sanchez & Stefani (1978) and, as will be shown later, could be blocked by D888. A Na^+ inward current could not be present under these conditions, since Na^+ was absent in the external solution. In addition, the Na^+ channels were blocked by TTX in the external solution. The slow Ca^{2+} inward current thus obtained was of the order of 40–120 nA. Assuming a fibre length of 1·3 mm and a fibre diameter of 50 μm an inward current of 20–60 $\mu A/cm^2$ was calculated, a value which is roughly of the same magnitude as that estimated by Sanchez & Stefani (1978), i.e. 81 $\mu A/cm^2$, with the same external Ca^{2+} concentration but in a hyperosmotic solution.

It can be seen that a repolarization of 15 s duration at −90 mV restored the main part of the Ca^{2+} current while the whole time course was slightly lengthened. An additional restoration time of 3 min did not further increase the Ca^{2+} current. A corresponding decrease in maximum Ca^{2+} current during the course of an experiment was generally observed, in particular when long-lasting depolarizing pulses were

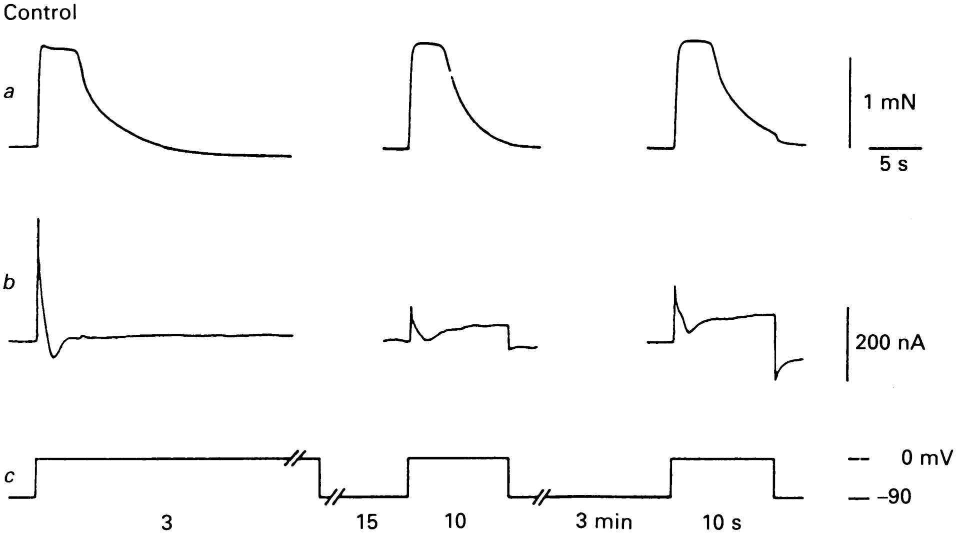

Fig. 10. Control experiment in the absence of the drug with force (*a*) and Ca^{2+} current (*b*) during a pulse programme (*c*) with a long-lasting initial depolarization. Solution C without Cd^{2+}; temperature = 10 °C.

applied. This phenomenon has been described as 'run-down' by other authors and explained as a protease-induced irreversible impairment of the Ca^{2+} channel (Hescheler & Trautwein, 1988). A quantitative analysis was further impeded (a) by continued increase in leakage current (the 'stabilizer' Cd^{2+} was absent and could not be applied because it blocks Ca^{2+} channels); (b) by movement artifacts from vigorously contracting fibres and (c) by an outward current at the onset of depolarization probably carried by K^+ through the delayed rectifier channel, which was obviously incompletely blocked by TEA.

Our programme was therefore restricted to a qualitative investigation of the behaviour of the Ca^{2+} current in paralysed muscle fibres. In Fig. 11*A*, the paralysed state was induced by depolarizing the fibre for 3·5 min in the presence of 0·5 μM-(−)-D888. A repolarization time of 1·5 min generated no restoration of force and also the Ca^{2+} current had not recovered after this time either. However, a subsequent hyperpolarization to −150 mV for 5 min eventually restored both force and Ca^{2+} current. In Fig. 11*B* using 0·2 μM-(−)-D888, an intermediate phase with a partial restoration of current and force can be seen. Similar results with a retarded parallel restoration of force and Ca^{2+} current were obtained in several further experiments with different concentrations of the (−)-isomer. The (+)-isomer was not tested.

These results were not unexpected. A similar retardation of restoration of the slow Ca^{2+} current by more than one order of magnitude had been described in cat

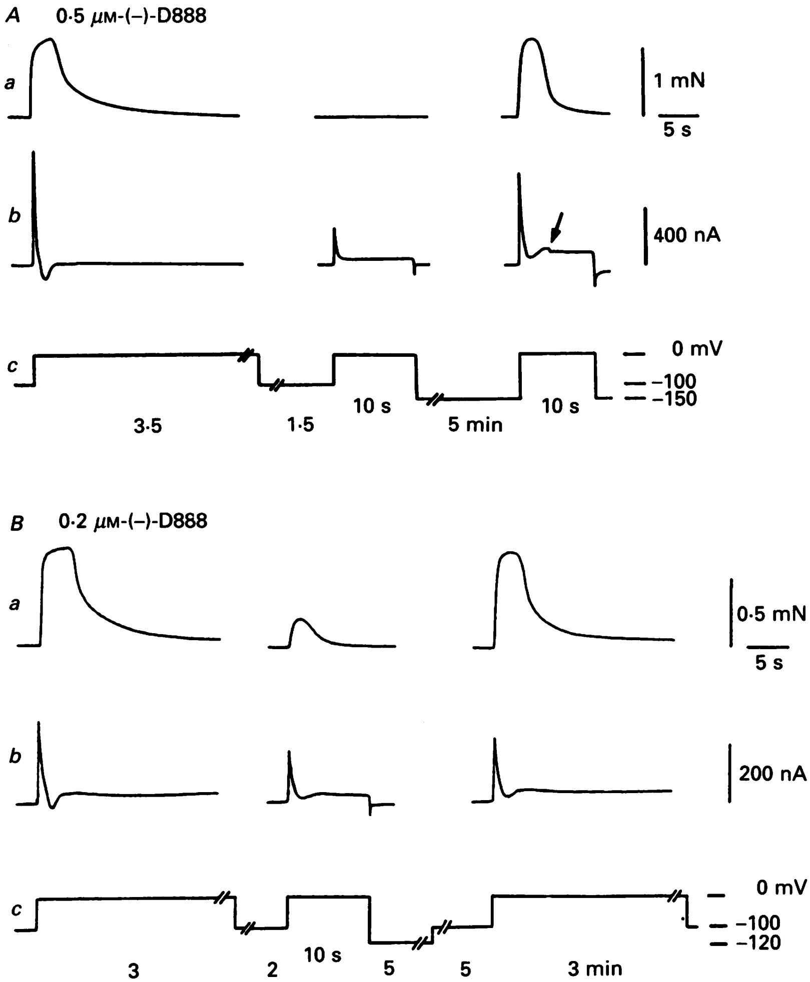

Fig. 11. Force (*a*) and Ca^{2+} current (*b*) during different pulse programmes (*c*) causing paralysis and restoration at 0·5 μM-(–)-D888 (*A*) and 0·2 μM-(–)-D888. (*B*). The arrow indicates a typical movement artifact in the current record. Two fibres; solution C without Cd^{2+}; temperature = 10 °C.

ventricular muscle in the presence of D600 by McDonald, Pelzer & Trautwein (1984). The equally slow restoration of current and force might suggest a causal relation between the two processes, in particular since it has recently been shown that the isolated Ca^{2+}-release channel in the reticulum can be activated by Ca^{2+} (Lai, Erickson, Rousseau, Liu & Meissner, 1988). However, the influx of Ca^{2+} can, if at all, only play a modulatory role in excitation–contraction (E–C) coupling. This arises from the observations that force becomes activated *before* the Ca^{2+} current commences and that it can still be induced when the Ca^{2+} current is blocked by Cd^{2+}, as shown in the present work, or when Ca^{2+} has been removed from the external solution (Lüttgau & Spiecker, 1979).

DISCUSSION

Comparison with the action of other phenylalkylamine derivatives

The present experiments revealed that the inhibitory action of desmethoxyverapamil (D888) on excitation–contraction coupling in skeletal muscle fibres is similar to that of methoxyverapamil (D600). Likewise it induced a paralysed state and caused a shift in the steady-state restoration curve to more negative potentials. However, D888 proved to be more effective than D600. With the racemate of D600, the voltage shift $\Delta\bar{V}$ was still insignificant at 0·5 μM and reached 32 mV at 5μM (Berwe *et al.* 1987). In the present experiments shifts of 20 and 42 mV were measured under slightly different conditions with 0·5 μM of the (+)- and (−)-isomer, respectively, and of 41 and 46 mV with 5 μM of either isomer. This difference in potency is comparable to that found by other authors who investigated different parameters. Thus, Nawrath & Raschack (1987) investigated the inhibition of a calcium-induced contraction in potassium-depolarized rat aortas and estimated a relative potency of 1 : 3·0 : 12·1 for the (−)-isomers of verapamil, D600 and D888, respectively.

Site of action

Berwe *et al.* (1987) suggested that D600 acts directly on the voltage sensor of force activation in the T-tubular membrane and proposed a state-dependent binding to the inactivated sensor. This model has been adopted here to interpret the results with D888. Our hypothesis of a direct interference of the phenylalkylamines with the control mechanism in the T-tubular membrane is primarily based on the observed potential dependence of their paralysing action. It was directly substantiated by measurements of the effect which D600 had on intramembrane charge movements, supposed to reflect the gating of the voltage sensor in the T-tubular membrane. Hui *et al.* (1984) were the first to show that in paralysed fibres intramembrane charge movements are 'immobilized'. Recently Melzer & Pohl (1987) extended this investigation by demonstrating that in the presence of D600 'restoration' of charge movement kinetics and Ca^{2+} release from the reticulum is retarded in a way comparable to that of force. Charge movement measurements in the presence of D888 have not been performed yet. However, considering the results with D600 it appears rather obvious that this substance induces the same effect.

In this context, it is of interest to note that a paralysed-like state also develops during long-lasting depolarizations in the absence of the drug (see Fig. 2). This process can be accelerated if external Ca^{2+} is replaced by Mg^{2+} (Lüttgau, Gottschalk & Berwe, 1986). It seems probable that phenylalkylamines promote a process which, under normal conditions, develops at a very slow rate in depolarized fibres (see Berwe *et al.* 1987).

The binding of D888 to the voltage sensor

The main part of the present work is the investigation of the shift in the steady-state potential dependence of force restoration to more negative potentials and the derivation of the dissociation constants for the two isomers by assuming a Boltzmann distribution of the sensor in the T-tubular membrane and a selective binding of the drug to the inactivated sensor. This has already been described in detail in the Results section. In Table 1, the outcome of the present work ascertained with electrophysiological methods is compared with that of binding studies carried out by different authors. The reasonable conformity between the two sets of results obtained with the help of different methods is encouraging. It strengthens the

TABLE 1. Comparison of the present K_D values derived from electrophysiological experiments in whole fibres with those obtained from binding studies with radiolabelled D888 in fibre fragments published by different authors

	Present data	Data from binding studies of skeletal muscle fibres	Citations
$K_{D(-)}$ (nM)	1·71	2·2	(Goll *et al.* 1984)*
		1·5	(Galizzi *et al.* 1986)*
		7·2	(Reynolds *et al.* 1986)†
$K_{D(+)}$ (nM)	12·9	17·0	(Goll *et al.* 1984)*
		3·0	(Galizzi *et al.* 1986)*
		54·2	(Reynolds *et al.* 1986)†

* Binding studies with T-tubular membranes.
† Binding studies with microsomes.

applicability of the present model but does not necessarily exclude alternative explanations. This is particularly true because the control of Ca^{2+} release exerted by the voltage sensor appears to be more complicated than had been formerly assumed. Recent charge movement experiments led to the suggestion of a multiple-state model (Melzer, Schneider, Simon & Szücs, 1986) or even of separate charging compounds (Huang, 1984). Considering these developments, our analysis, which rests on a simple Boltzmann distribution of charged sensors in the T-tubular membrane, can only be regarded as a first approximation to a more complex process.

Binding studies with (−)-[^{3}H] D888 in purified T-tubular membranes by different groups (Goll *et al.* 1984; Galizzi, Borsotto, Barhanin, Fosset & Lazdunski, 1986) revealed a slow association of this drug to its receptor and likewise a slow dissociation. Galizzi *et al.* (1986), for example, estimated a dissociation rate constant of $3{\cdot}5 \times 10^{-4}$ s^{-1} (half-time 33 min; temperature = 10 °C) and an association rate constant of 3×10^5 M^{-1} s^{-1}. The authors explained the slowness in binding by suggesting a conformational change of the ligand–receptor complex which might succeed a first bimolecular association step. The slow transition into the paralysed state and the slow restoration of paralysed fibres as observed here manifest in our mind the slow association and dissociation observed in the cited binding studies.

The effect of Ca^{2+} antagonists on Ca^{2+} channel and voltage sensor

D888 caused a delay in restoration of both voltage sensor and Ca^{2+} channel. Although the action of this drug on the Ca^{2+} channel has not yet been worked out quantitatively, it appears reasonable to assume that the mode of action of D888 and other phenylalkylamine derivatives is the same in both systems. Diltiazem, a prototype of another class of Ca^{2+} antagonists (benzothiazepines), also causes a delay in restoration of channel and sensor (Böhle, Gottschalk, Neuhaus & Lüttgau, 1987;

T. Böhle, unpublished observations). Less understood is the mode of action of dihydropyridines, a third major class of Ca^{2+} antagonists (e.g. nifedipine). Dihydropyridines bind to numerous T-tubular binding sites with a K_D of ~ 1 nM, whereby negative potentials partially inhibit binding in a non-competitive fashion (Schwartz, McCleskey & Almers, 1985). They again influence both, the Ca^{2+} channel (e.g. Flockerzi, Oeken, Hofmann, Pelzer, Cavalié & Trautwein, 1986) and the voltage sensor (Rios & Brum, 1987). However, nifedipine, one of the best-known dihydropyridine derivatives, has little or no effect on charge movements (Lamb & Walsh, 1987; Rios & Brum, 1987) and force (Neuhaus, 1986) induced from negative holding potentials. This proved to be true, even at micromolar concentrations, while with the same concentration Ca^{2+} channels are completely blocked.

The interference of the three main classes of Ca^{2+} antagonists with both sensor and channel, probably each at a specific binding site (cf. Glossmann & Striessnig, 1988), suggests that the two systems are structurally related or even identical, as has recently been proposed by Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kangawa, Kojima, Matsuo, Hirose & Numa (1987). At present the experimental evidence neither confirms nor refutes the latter assumption. However, different threshold potentials and time courses of activation (the activation of the Ca^{2+} channel is delayed in skeletal muscle fibres) suggest that there are at least two rather independent gating mechanisms. This assumption has recently been substantiated by Feldmeyer & Lüttgau (1988) who showed that 10 mM-perchlorate shifted the potential dependence of the activation of intramembrane charges and force by 20 mV to more negative potentials, while the threshold for the activation of the Ca^{2+} channel remained unchanged.

We wish to thank Dr W. Melzer for helpful suggestions regarding the theoretical part of the present work and for carrying out the fitting program. The isomers of D888 were a gift from Knoll AG, Ludwigshafen (FRG). Supported by the Deutsche Forschungsgemeinschaft (Forschergruppe KONZELL).

REFERENCES

Bean, B. P. (1984). Nitrendipine block of cardiac calcium channels: High affinity binding to the inactivated state. *Proceedings of the National Academy of Sciences of the USA* **81**, 6388–6392.

Berwe, D. & Feldmeyer, D. (1984). Investigation of force restoration in short toe muscle fibres under voltage clamp control. *Pflügers Archiv* **402**, R46.

Berwe, D., Gottschalk, G. & Lüttgau, H. Ch. (1987). Effects of the calcium antagonist gallopamil (D600) upon excitation–contraction coupling in toe muscle fibres of the frog. *Journal of Physiology* **385**, 693–707.

Böhle, T., Gottschalk, G., Neuhaus, R. & Lüttgau, H. Ch. (1987). The effects of Ca antagonists upon excitation–contraction coupling in skeletal muscle fibres. *Pflügers Archiv* **408**, R79.

Brum, G., Fitts, R., Pizarro, G. & Rios, E. (1988). Voltage sensors of the frog skeletal muscle membrane require calcium to function in excitation–contraction coupling. *Journal of Physiology* **398**, 475–505.

Brum, G., Rios, E. & Stefani, E. (1988). Effects of external calcium on calcium movements of excitation–contraction coupling in frog skeletal muscle fibres. *Journal of Physiology* **398**, 441–473.

Cota, G. & Stefani, E. (1986). A fast-activated inward calcium current in twitch muscle fibres of the frog (*Rana montezume*). *Journal of Physiology* **370**, 151–163.

Eisenberg, R. S., McCarthy, R. T. & Milton, R. L. (1983). Paralysis of frog skeletal muscle fibres by the calcium antagonist D-600. *Journal of Physiology* **341**, 495–505.

ERDMANN, R., LÜTTGAU, H. CH. & MELZER, W. (1988). The effect of Devapamil (D888) on force and Ca current in isolated frog skeletal muscle fibres. *Journal of Physiology* **407**, 73*P*.

FELDMEYER, D. & LÜTTGAU, H. CH. (1988). The effects of perchlorate on Ca current and mechanical force in frog skeletal muscle fibres. *Pflügers Archiv* **411**, R190.

FLOCKERZI, V., OEKEN, H. J., HOFMANN, F., PELZER, D., CAVALIÉ, A. & TRAUTWEIN, W. (1986). Purified dihydropyridine-binding site from skeletal muscle t-tubules is a functional calcium channel. *Nature* **323**, 66–68.

GALIZZI, J.-P., BORSOTTO, M., BARHANIN, J., FOSSET, M. & LAZDUNSKI, M. (1986). Characterization and photoaffinity labeling of receptor sites for the Ca^{2+} channel inhibitors *d-cis*-diltiazem, (±)-bepridil, desmethoxyverapamil, and (+)-PN 200–110 in skeletal muscle transverse tubule membranes. *Journal of Biological Chemistry* **261**, 1393–1397.

GLOSSMANN, H. & STRIESSNIG, J. (1988). Calcium channels. *Vitamins and Hormones* **44**, 155–328.

GOLL, A., FERRY, D. R., STRIESSNIG, J., SCHOBER, M. & GLOSSMANN, H. (1984). (−)-[^{3}H]Desmethoxyverapamil, a novel Ca^{2+} channel probe. *FEBS Letters* **176**, 371–377.

GOMOLLA, M., GOTTSCHALK, G. & LÜTTGAU, H. CH. (1983). Perchlorate-induced alterations in electrical and mechanical parameters of frog skeletal muscle fibres. *Journal of Physiology* **343**, 197–214.

HESCHELER, J. & TRAUTWEIN, W. (1988). Modification of cardiac calcium current by intracellularly applied trypsin. *Pflügers Archiv* **411**, R27.

HILLE, B. (1977). Local anesthetics: Hydrophilic and hydrophobic pathways for the drug–receptor reaction. *Journal of General Physiology* **69**, 497–515.

HUANG, C. L.-H. (1984). Analysis of 'off' tails of intramembrane charge movements in skeletal muscle of *Rana temporaria*. *Journal of Physiology* **356**, 375–390.

HUI, C. S., MILTON, R. L. & EISENBERG, R. S. (1984). Charge movement in skeletal muscle fibers paralysed by the calcium-entry blocker D-600. *Proceedings of the National Academy of Sciences of the USA* **81**, 2582–2585.

LAI, F. A., ERICKSON, H. P., ROUSSEAU, E., LIU, Q. Y. & MEISSNER, G. (1988). Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature* **331**, 315–319.

LAMB, G. D. & WALSH, T. (1987). Calcium currents, charge movement and dihydropyridine binding in fast- and slow-twitch muscles of rat and rabbit. *Journal of Physiology* **393**, 595–617.

LÜTTGAU, H. CH. (1970). Caffeine, calcium and the activation of contraction. In *Calcium and Cellular Function*, ed. CUTHBERT, A. W., pp. 241–248. London: MacMillan & Co. Ltd.

LÜTTGAU, H. CH., GOTTSCHALK, G. & BERWE, D. (1986). The role of Ca^{2+} in inactivation and paralysis of excitation–contraction coupling in skeletal muscle. In *Fortschritte der Zoologie*, vol. 33, *Membrane Control of Cellular Activity*, ed. LÜTTGAU, H. CH., pp. 195–203. Stuttgart: Gustav Fischer Verlag.

LÜTTGAU, H. CH. & SPIECKER, W. (1979). The effects of calcium deprivation upon mechanical and electrophysiological parameters in skeletal muscle fibres of the frog. *Journal of Physiology* **296**, 411–429.

McDONALD, T. F., PELZER, D. & TRAUTWEIN, W. (1984). Cat ventricular muscle treated with D600: Characteristics of calcium channel block and unblock. *Journal of Physiology* **352**, 217–241.

MELZER, W. & POHL, B. (1987). Effects of D600 on the voltage sensor for Ca release in skeletal muscle fibres of the frog. *Journal of Physiology* **390**, 151*P*.

MELZER, W., SCHNEIDER, M. F., SIMON, B. J. & SZÜCS, G. (1986). Intramembrane charge movement and calcium release in frog skeletal muscle. *Journal of Physiology* **373**, 481–511.

MORE, J. J., GARBOW, B. S. & HILLSTROM, K. E. (1980). User guide for MINPACK-I, Anl-80-74. Argonne National Laboratory, Argonne.

NAWRATH, H. & RASCHACK, M. (1987). Effects of (−)-Desmethoxyverapamil on heart and vascular smooth muscle. *Journal of Pharmacology and Experimental Therapeutics* **242**, 1090–1097.

NEUHAUS, R. (1986). The effect of nifedipine on slow Ca^{2+} inward current and force development in isolated skeletal muscle fibres of the frog. *Journal of Physiology* **382**, 122*P*.

REYNOLDS, I. A., SNOWMAN, A. M. & SNYDER, S. H. (1986). (−)-[^{3}H]Desmethoxyverapamil labels multiple calcium channel modulator receptors in brain and skeletal muscle membranes: Differentiation by temperature and dihydropyridines. *Journal of Pharmacology and Experimental Therapeutics* **237**, 731–738.

RIOS, E. & BRUM, G. (1987). Involvement of dihydropyridine receptors in excitation–contraction coupling in skeletal muscle. *Nature* **325**, 717–720.

ROUSSEAU, E., LADINE, J., LIU, Q.-Y. & MEISSNER, G. (1988). Activation of the Ca release channel of skeletal muscle sarcoplasmic reticulum by caffeine and related compounds. *Archives of Biochemistry and Biophysics* **267**, 75–86.

SANCHEZ, J. A. & STEFANI, E. (1978). Inward calcium current in twitch muscle fibres of the frog. *Journal of Physiology* **283**, 197–209.

SCHWARTZ, L. M., MCCLESKEY, E. W. & ALMERS, W. (1985). Dihydropyridine receptors in muscle are voltage-dependent but most are not functional calcium channels. *Nature* **314**, 747–751.

SIEBLER, M. & SCHMIDT, H. (1987). D600 prolongs inactivation of the contractile system in frog twitch fibres. *Pflügers Archiv* **410**, 75–82.

TANABE, T., TAKESHIMA, H., MIKAMI, A., FLOCKERZI, V., TAKAHASHI, H., KANGAWA, K., KOJIMA, M., MATSUO, H., HIROSE, T. & NUMA, S. (1987). Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature* **328**, 313–318.